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# *In vitro* replication through nucleosomes without histone displacement

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A well-characterized set of proteins encoded by bacteriophage T4 replicates DNA *in vitro* and generates replication forks that can pass nucleosomes. The histone octamers remain associated with newly replicated DNA even in the presence of excess DNA competitor, and intact nucleosomes re-form on the two daughter DNA helices. It is concluded that nucleosomes are designed to open up transiently to allow the passage of a replication fork without histone displacement.

THE fundamental unit of eukaryotic chromatin is the nucleosome—a disc-like structure composed of two each of the four histones $H_2A$, $H_2B$, $H_3$ and $H_4$, and ~145 base pairs (bp) of DNA wrapped around the outside of this octameric histone core. The histones condense the DNA by forming nucleosomes and higher-order structures. In addition, the variability in histone structure and modification allow the formation of different types of nucleosomes and therefore different states of chromatin (for a review, see ref. 1). These different chromatin states are thought to have a role in determining the transcriptional capability of specific chromosomal regions (for a review, see ref. 2). Each time a cell divides, it is not just the DNA that must be replicated, but the entire complex structure of the chromatin. Several studies indicate that specific features of transcriptionally competent chromatin are reproduced behind the replication fork on both daughter strands[3–5]. How this is accomplished is not known. In principle, the parental histones could be directly inherited as the replication fork passes, and these old histones could then serve as a template to help define the post-replicative distribution of the new ones[6–9]. Indeed, there is evidence that the parental histone octamers are not disrupted by the replication process and persist in cells through several generations[8,10,11]. But attempts to determine the mode of segregation of the parental histones to the replicated daughter strands

have given contradictory results. Some data support a conservative segregation of all of the parental histones to the daughter DNA helix made on the leading-strand of the fork, where DNA is synthesized continuously[10,12–14]. Other data indicate, however, that the histone octamers are distributed to both of the daughter helices behind the fork[11,15–22]. For example, Sogo *et al.*[22] have used electron microscopy to study nucleosome segregation during simian virus 40 (SV40) DNA replication by blocking new histone synthesis with cycloheximide and then using psoralen crosslinking to mark the positions of the old nucleosomes inherited behind the fork *in vivo*. Under these conditions, nucleosomes were seen to be inherited in small clusters that were equally distributed on both daughter DNA helices. Because these nucleosomes were not present immediately behind the fork, it was proposed that the histone octamers dissociate transiently as the replication fork passes, but quickly reassociate with the newly replicated DNA.

Do the histones remain permanently associated with the DNA or are they transiently removed during the passing of the fork? We have now addressed this question by directly studying what happens to a nucleosome when a replication fork passes through it *in vivo*. We began by assessing the ability of the highly purified *in vitro* T4 bacteriophage DNA replication system to replicate through nucleosomes. We found that the T4 replication fork can replicate past histone octamers on DNA. This allowed us to determine the fate of these histones during the replication process with a completely defined system that contains only nine highly purified T4 replication proteins, the four nucleosomal histones and a purified DNA template.

## The artificial chromatin template

The nucleosome-containing DNA template that we used for replication with the T4 *in vitro* replication system is a 4.7-kilobase (kb) circular plasmid DNA containing the replication origin of bacteriophage M13. Nicking this origin with the bacteriophage gene 2 protein provides a unique 3' end that serves as a starting site for initiation of *in vitro* DNA synthesis[23]. Unfortunately, this origin region is also a preferential site for nucleosome assembly. To preserve the accessibility of the cutting site of the gene 2 protein, we restricted nucleosome assembly on this template to only a few nucleosomes per DNA molecule. We added nucleosomes to more or less random positions on

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the template by transferring ^{3}H-lysine-labelled histone octamers from a nucleosome donor (the unusually stable nucleosome reconstituted on a DNA fragment containing a 5S ribosomal RNA gene)[24] to the circular supercoiled form of the template DNA molecule.

The reconstituted nucleosomes seem to be authentic in that they contain a full set of histones (Fig. 1a), with the same relative composition of the four histones as the original donor nucleosomes. Also, digestion of the nucleosome-containing DNA templates with micrococcal nuclease produces a DNA fragment of ~145 bp, similar in size to the DNA fragment released from native nucleosome cores (Fig. 1b). The specific activity of the reconstituted DNA–histone complexes (~13,000 c.p.m. $\mu g^{-1}$) indicates the transfer of about three histone octamers per DNA template. This value is in agreement with the number of nucleosomes observed by electron microscopy for most of the DNA templates (Fig. 1c). But we found some template molecules with no nucleosomes, whereas others contained up to six or seven nucleosomes.

## The fork can pass nucleosomes after a pause

Seven bacteriophage T4-encoded proteins reconstitute an *in vitro* DNA replication system that catalyses coupled leading- and lagging-strand DNA synthesis at a replication fork[25]. The proteins involved are the T4 DNA polymerase holoenzyme (the products of T4 genes *43*, *44/62* and *45*), a helix-destabilizing

single-stranded binding (SSB) protein (gene *32* protein) and the T4 primosome, which is composed of a DNA helicase (gene *41* protein) and a DNA primase (gene *61* protein)[26-36].

In our initial experiments, we found that the presence of nucleosomes on the DNA blocks replication catalysed by the T4 replication proteins. But nucleosomes can be bypassed by the T4 replication fork, provided that the T4 dda (DNA-dependent ATPase) protein is present. The dda protein is a DNA helicase that removes an RNA polymerase molecule or an operator-bound gene-repressor protein from the DNA template ahead of the fork[23,37,38]. An additional T4 protein was included in the *in vitro* DNA replication system: the T4 59 protein. This protein, which has been recently purified and characterized, greatly facilitates the loading of an active primosome onto the replication fork, ensuring efficient lagging-strand, as well as leading-strand, DNA synthesis (J. Barry and B.A., manuscript in preparation).

On a circular template that lacks nucleosomes, replication proceeds in a 'rolling-circle' mode that allows many rounds of copying[39]. Figure 2 shows that the leading-strand products are long DNA strands that move slowly during alkaline agarose gel electrophoresis, whereas the lagging-strand products accumulate as a heterogeneous smear of Okazaki fragments of smaller size (Fig. 2, third lane from left).

In the presence of nucleosomes, leading-strand DNA products of unusually short length were produced in the first few minutes

FIG. 1 Characterization of an artificial chromatin template. The plasmid pJMC110 DNA to which radioactive nucleosomes were transferred was purified by centrifugation through a sucrose gradient. After the peak fractions were pooled, concentrated, and dialysed into storage buffer, the nucleosomes on the DNA were characterized. *a*, Histone composition. Aliquots were subjected to SDS-PAGE on a 13.5% gel. Proteins were identified by autoradiography. Lane 1, initial H1-depleted native nucleosomes prepared from Chinese hamster ovary (CHO) cells; lane 2, nucleosome reconstituted on the DNA fragment containing the 5S rRNA gene (nucleosome donor); lane 3, nucleosomes on the artificial chromatin template (nucleosome acceptor). *b*, DNA protected from micrococcal nuclease digestion. After addition of $CaCl_2$ to 3 mM the artificial chromatin template was digested with micrococcal nuclease at 37 °C for 2 and 4 min. The DNA was then purified, end-labelled, and electrophoresed through an 8% polyacrylamide gel. The numbers to the right of the autoradiograph indicate the size of DNA fragment markers that were electrophoresed as standards in the same gel. *c*, Electron microscopy. The sample was fixed in 0.1% glutaraldehyde at 4 °C[57] and processed as described in ref. 58. Bar, 0.1 $\mu$m.

METHODS. To prepare a DNA template containing nucleosomes, labelled histone octamers were transferred to plasmid pJMC110 DNA by a low salt exchange method, as described in ref. 59. The histone donor was a nucleosome reconstituted on a 256-bp DNA fragment containing a sea urchin 5S rRNA gene which forms an exactly positioned core particle[24]. Because of the unusual stability of the nucleosome on this DNA sequence, nucleosome dissociation, which leads to nonspecific binding of the histones to the acceptor DNA, was largely prevented during the exchange process. To prepare this histone donor, the 256-bp DNA fragment containing the 5S rRNA gene (40 $\mu$g $ml^{-1}$) was mixed with a 10-fold excess of H1-depleted nucleosome core particles[50], containing ^{3}H-labelled histone (prepared from ^{3}H-lysine-labelled CHO cells; core particle specific activity, 140,000 c.p.m. $\mu g^{-1}$), at a DNA concentration of 400 $\mu$g $ml^{-1}$ in a high-salt buffer (10 mM Tris-HCl buffer, ph 7.4, 1 mM $Na_3$EDTA, 2 mM DTT, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 0.8 M NaCl and 100 $\mu$g $ml^{-1}$ human serum albumin (HSA)). Histone octamer exchange was performed by incubating this mixture for 20 min at 37 °C. The salt concentration was then decreased at 37 °C by two successive dilutions to 600 mM NaCl (100-mM steps every 10 min). The reconstituted nucleosome was then separated from the slower-sedimenting initial core particles by centrifugation through a sucrose gradient containing 0.55 M NaCl and 100 $\mu$g $ml^{-1}$ HSA. The efficiency of reconstitution and purification was monitored by electrophoresis through a 6% polyacrylamide gel (acrylamide/methylene-bis-acrylamide ratio
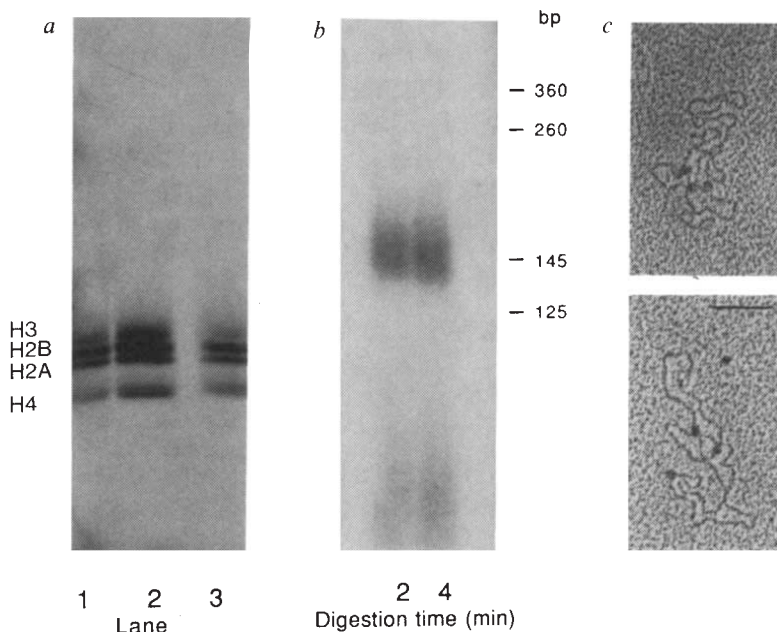


of 46:1) as described in ref. 60. At least 90% of the DNA fragment containing the 5S rRNA sequence was reconstituted into nucleosomes, which contained the correct stoichiometry of the four core histones. Fractions containing these reconstituted nucleosomes were dialysed against 10 mM Tris-Cl buffer, pH 7.4, 1 mM $Na_3$EDTA, 2 mM DTT, 0.1 mM PMSF, 0.1 M NaCl and concentrated to ~100 $\mu$g $ml^{-1}$ DNA using a collodion bag apparatus. The reconstituted nucleosomes were stored at 4 °C in the presence of 0.02% sodium azide. No dissociation or proteolytic degradation of the histones was detected during these processes. The subsequent transfer of the histone octamers from the 5S rRNA gene to supercoiled plasmid pJMC110 DNA was performed by a similar method. The supercoiled DNA was incubated with the purified nucleosomes at molar ratios of 1:6 and 1:10 (plasmid:nucleosome). The exchange reaction was stopped by decreasing the salt concentration to 100 mM NaCl (100-mM steps every 10 min to 500 mM NaCl, followed by dilutions to 250 mM and 100 mM NaCl). The nucleosome-containing DNA molecule was then separated from the much smaller donor nucleosome either by centrifugation through a sucrose gradient or by chromatography on a Sepharose 4B gel filtration column in the presence of 100 $\mu$g $ml^{-1}$ HSA.

of the reaction, indicating that the replication forks that had assembled moved more slowly than those that assembled when no nucleosomes were present on the DNA template. The replication forks were, however, able to pass a nucleosome after a pause. After 2 min of reaction, many of the replication forks had performed more than one round of synthesis on the chromatin template (leading strand >9.4 kb, see Fig. 2, rightmost lane), and by 5 min nearly all of the forks had done so (data not shown).

## Histone octamers remain on the DNA

If the nucleosomes are knocked off the circular DNA template molecule every time the T4 replication fork passes, as is the *Escherichia coli* RNA polymerase molecule bound to a promoter[23], the leading-strand DNA polymerase molecule would encounter nucleosomes only during its first round of rolling-circle DNA synthesis (see Fig. 3a and below). In this case, all pausing of the replication fork on the nucleosome-containing template should disappear once the size of the leading strand reaches 9.4 kb (twice the size (4.7 kb) of the DNA circle). Examination of the time course of many experiments like the one of Fig. 2 reveals that strong pausing also occurs during the second round of rolling-circle DNA replication (leading-strand size between 9.4 and 14.1 kb). At least some of the nucleosomes must therefore remain on the DNA circle after the fork passes. But the extent of pausing clearly decreases as the leading strand grows, arguing against the model in which all of the histone octamers segregate as intact nucleosomes to the leading strand[13].

A trivial interpretation of our results is that histone octamers are in fact knocked off the DNA by the moving fork, but very rapidly rebind to any DNA molecules in the solution (either as octamers or as smaller histone complexes). To test this hypothesis, we performed a series of *in vitro* replication experiments in the presence of a large excess of nonreplicating DNA that lacked nucleosomes. If nucleosomes transiently dissociate, then this excess DNA should act as a 'trap' and rebind the dissociated histones, thereby altering their distribution. In this way, we could determine whether histone octamers are directly inherited by the daughter DNA molecules at the fork, or whether they transiently become free in solution. Under the physiological salt conditions (120 mM potassium acetate) that we used in these replication experiments, nucleosomes on the chromatin template do not transfer onto the added DNA spontaneously[2].

We designed the experiment illustrated diagrammatically in Fig. 3a to test whether ^{3}H-labelled histone octamers are released from an artificial chromatin template as a result of replication. Replicated DNA is readily discriminated from unreplicated DNA (including both unused chromatin templates and competitor DNA) by its resistance to the enzyme *Dpn*1. The replicated DNA molecules are separated from the digested unreplicated DNA on a Sepharose Cl-2B gel-filtration column. Figure 3b presents the result of such an experiment, performed both with and without a 10-fold excess of competitor DNA present during the DNA replication process. Experiments in which the replicated DNA was labelled with [^{32}P]dTTP show that all of the replicated molecules fractionate into the excluded peak (fractions 23–26). A large peak of ^{3}H-labelled histones coeluted with this *Dpn*1-resistant newly synthesized DNA; the rest of the histones eluted later from the column, as expected from their presence on the substantial fraction of unreplicated DNA circles. Most importantly, the amount of ^{3}H-labelled histone that eluted with the replicated DNA was insensitive to the presence of a 10-fold excess of competitor DNA (Fig. 3b), demonstrating that the histones are not released even transiently from the DNA during the passage of the replication fork. As expected, when the T4 DNA polymerase was omitted in the

FIG. 2 The effect of nucleosomes on replication fork movement *in vitro.* DNA synthesis was performed using either naked DNA (two lanes on the left(−nuc)) or artificial chromatin (two lanes on the right (+nuc)) as the template. The radioactive DNA product strands were sized by alkaline agarose gel electrophoresis at 1 and 2 min after the start of DNA synthesis. The total nucleotide incorporation into DNA was reduced four-fold by the presence of nucleosomes; to facilitate comparison of product sizes, twice as much reaction mix was analysed for the two lanes on the right. The indicated position of the primer strand after one round of rolling-circle replication (9.4 kb pairs) was determined from the migration rates of DNA markers (lane M). Because the replication forks proceeded for only a short distance on the nucleosome-containing templates (+nuc), most of these forks had not yet initiated Okazaki fragment synthesis (see Fig. 5 for results after further incubation).

METHODS. To provide a primer to start DNA synthesis on the plasmid pJMC110 DNA template, the DNA was specifically nicked at the M13 bacteriophage gene 2 protein recognition site. One unit of bacteriophage fd gene 2 protein[61] was incubated with 0.5 μg DNA in 20 mM Tris-HCl buffer, pH 8.5, 80 mM NaCl, 2.5 mM MgCl$_2$, 1 mM β-mercaptoethanol, 5% glycerol at 30 °C for 2.5 min. The reaction was terminated by addition of Na$_3$EDTA to 3 mM and cooling the reaction mixture to 4 °C. Analysis of the products of this reaction by agarose gel electrophoresis showed that ~90% of the supercoiled DNA was converted to open circular form in the reaction. But when a chromatin template was treated in this way, the percentage of open circular form produced was reduced if more than a few nucleosomes were reconstituted on each plasmid DNA molecule. *In vitro* DNA replication was performed in the presence of 33 mM Tris–acetate (pH 7.8), 120 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM DTT, 1 mM ATP, 1 mM GTP, 0.1 mM UTP, 0.1 mM CTP, 0.5 mM dATP, 0.5 mM dGTP, 0.15 mM dCTP, 0.08 mM [α-^{32}P]TTP, 5 μg ml$^{-1}$ template DNA, in the presence of 100 μg ml$^{-1}$ nuclease-free HSA as a protein carrier. In these reactions, the following T4 DNA replication proteins were present at the indicated concentrations: 2 μg ml$^{-1}$ T4 DNA polymerase, 50 μg ml$^{-1}$ T4 gene 32 protein, 20 μg ml$^{-1}$ T4 gene 44/62 protein, 18 μg ml$^{-1}$ T4 gene 45 protein, 20 μg ml$^{-1}$ T4 gene 41 protein, 1 μg ml$^{-1}$ T4 gene 61 protein, 10 μg ml$^{-1}$ T4 dda protein and 1 μg ml$^{-1}$ T4 59 protein. Partial synchronization of the replication reactions on the specifically nicked plasmid pJMC110 DNA tem-
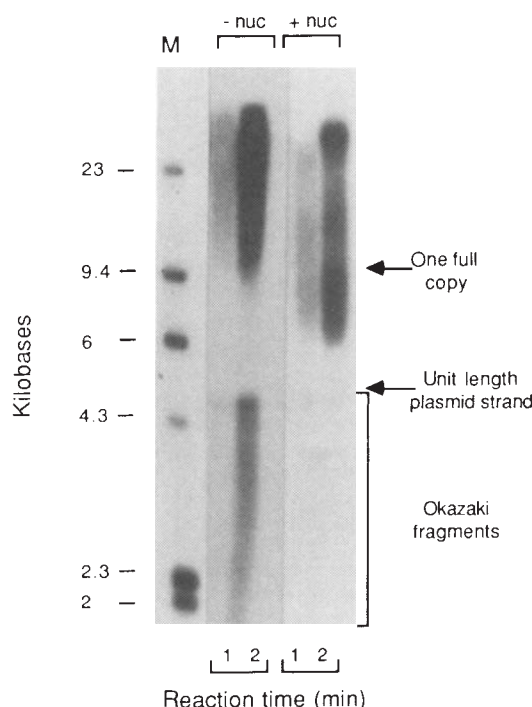


plate was achieved by preincubation in the absence of dCTP before allowing extensive DNA synthesis to begin by adding dCTP. The radioactive products of the reaction were then analysed by separating them by electrophoresis through a 0.6% agarose gel run in 30 mM NaOH, 1 mM Na$_3$EDTA, and autoradiographing the dried gel[23].

replication reaction (dotted line), ~95% of the ³H-labelled histones coeluted with the small DNA fragments produced by Dpn1 treatment (Fig. 3b, fractions 32-60).

Table 1 presents the results from several experiments identical to the experiment of Fig. 3b. In general, 30-35% of the ³H-labelled histone radioactivity was found associated with newly synthesized DNA in fractions 23-26, and the competitor DNA had no effect on this result. We conclude that among the DNA template molecules nicked by treatment with the gene 2 protein (~50% of the DNA templates), most, but not all, are used as a template by the T4 replication proteins.

We next examined whether all four of the individual histones remain associated with the replicated DNA molecules. For histones $H_2B$, $H_2A$ and $H_4$, the ratio of histones was the same as before replication (Fig. 4a; unfortunately, although H3 was present, its specific activity was too low to detect it accurately by fluorography). In addition, we digested the purified replicated DNA with micrococcal nuclease to test for DNA that was resistant to digestion because of the presence of nucleosomes. Protected DNA fragments with a size identical to that of fragments protected in a nucleosome core particle were observed (Fig. 4b). With further digestion, DNA fragments of a smaller size appeared, which were due to internal cuts in the nucleosome by micrococcal nuclease (lane 5).

We also attempted to examine the effect of replication on the structure of the nucleosome by electron microscopy. The electron micrographs indicated that histones that are bound to replicated DNA retain the basic nucleosomal structure. Globular particles morphologically similar to native nucleosomes were found associated with both the long double-stranded DNA tail

TABLE 1 Effect of 10-fold excess competitor DNA on retention of histones

| Experiment | % Newly made DNA in peak | % Histone in peak 1 (no DNA competitor) | % Histone in peak 1 (+DNA competitor) |
|---|---|---|---|
| 1 | 98 | — | — |
| 2 | — | 35 | 30 |
| 3 | — | 30 | 32 |
| 4 | — | 30 | 35 |
| 5 | — | — | 30 |

A 10-fold excess of competitor DNA has no effect on the retention of histones on replicated DNA molecules. DNA synthesis was allowed to occur for 5 to 10 min. The procedure described in Fig. 3a was then used to separate replicated DNA molecules, as in Fig. 3b. The Dpn1-resistant DNA fractions in peak 1 contain replicated DNA molecules, which produced an average of 3-4 template copies by rolling-circle replication (see Fig. 3a). Either ³²P-labelled DNA or ³H-labelled histones were monitored, depending on the component that was radioactive. The presence of the 10-fold excess of competitor DNA did not affect the efficiency or rate of replication of the chromatin template (data not shown).

and with the circular template (Fig. 5a). But we did not find this method to be a good way of analysing nucleosome behaviour during replication, because only a very small fraction of the replicated molecules were sufficiently spread for scoring. In addition, a significant amount of histone octamer dissociation occurred during the preparation steps required for electron microscopy.
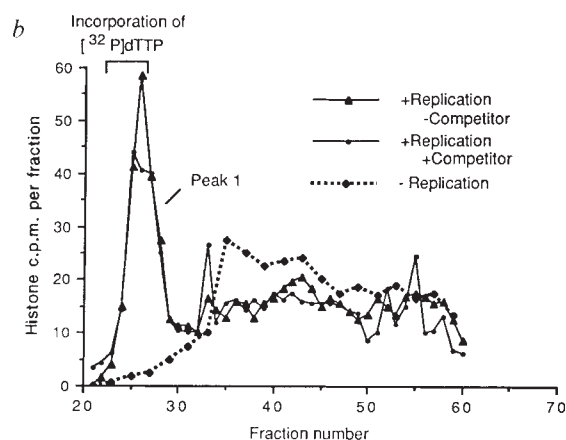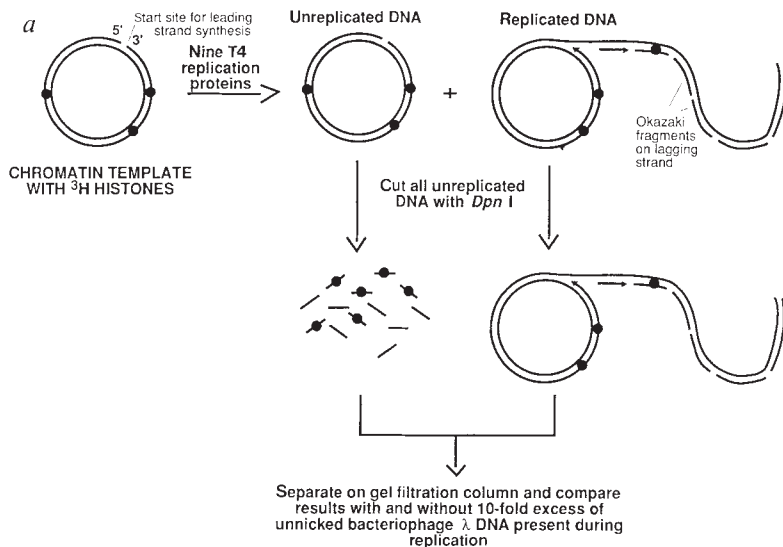


FIG. 3 The use of competitor DNA (83 μg ml⁻¹) to test for the direct transfer of histones from parental to daughter DNA helices at a DNA replication fork. DNA synthesis was performed with the complete T4 DNA replication system containing the nine proteins, as described in the text (see also Fig. 2). a, Schematic diagram of the experiments. In rolling-circle DNA replication, the leading strand DNA polymerase molecule moves continuously around the unnicked strand of the original circular DNA template molecule. As it moves, a single strand of DNA is displaced from the circle as a long linear 'tail'. All of the DNA product on the tail is synthesized by the lagging-strand DNA polymerase molecule, which produces a series of Okazaki fragments that remained unsealed in our experiments. In the T4 system, these fragments are very heterogeneous in length (400-4,000 nucleotides)²⁵. Because the plasmid pJMC110 DNA was produced in dam⁺ bacteria, it contains 22 fully methylated GATC sequences, which are cut by Dpn1. The hemimethylated DNA produced by replication in vitro becomes resistant to Dpn1 cleavage⁶². After Dpn1 treatment, the replicated molecules are separated from the Dpn1-degraded unreplicated plasmid DNA by chromatography on a Sepharose Cl-2B column. To test for the dissociation of labelled histones from the nucleosome-containing DNA template during replication, a 10-fold excess of linear DNA molecules was added as competitor to rebind any histones released during the replication reaction. This competitor DNA was

prepared by digesting bacteriophage λ DNA with BstEII and EcoRV, which produces fragments of 1.0 to 3.5 kb that do not replicate and can be readily separated from the much larger replicated molecules by Sepharose Cl-2B gel filtration. b, Gel filtration analysis of DNA-bound ³H labelled histones after replication of the artificial chromatin template in the presence or absence of competitor DNA. Replication was performed with 1 μg template and 10 μg competitor DNA in a total volume of 120 μl. After 10 min of replication at 37 °C, the salt concentration was raised to 190 mM NaCl. Twenty units of Dpn1 were then added for 20 min at 37 °C. After addition of 20 mM Na₃EDTA, the reaction products were chromatographed on a Sepharose Cl-2B gel filtration column (30 × 0.5 cm) run at a flow rate of 0.5 ml h⁻¹ in Tris-HCl buffer, pH 7.5, 1 mM Na₃EDTA, 10 mM NaCl at room temperature. Elution is from left to right, with fraction volumes of 0.15 ml. Aliquots from each fraction were counted to determine the distribution of the ³H radioactivity. In control experiments, an aliquot of the same chromatin template replicated in the presence of ³²P-labelled TTP was analysed to determine the elution profile of newly replicated DNA (marked with a bracket). The dotted line shows that no histones appear in peak 1 if DNA replication is blocked by omission of the T4 DNA polymerase (gene 43 protein) from the reaction mixture.

TABLE 2   Quantitation of electron-microscopy data

Size of single-stranded bubble (nucleotides): from ref. 22, $160 \pm 35$; unreplicated, $147 \pm 37$; replicated circle, $150 \pm 35$; replicated tail, $145 \pm 30$.

Partitioning of nucleosomes between circle and tail

| No. of template copies | No. of DNA molecules | Total nucleosome no. on circle | Total nucleosome no. on tail | Total nucleosome ratio tail: circle* | Average no. of nucleosomes per circle | Average no. of nucleosomes per tail |
|---|---|---|---|---|---|---|
| 0.6–1 | 6 | 26 | 8 | 0.30  (0.25) | 4.3 | 1.3 |
| 1–2 | 15 | 28 | 17 | 0.6   (0.52) | 1.8 | 1.1 |
| 2–3 | 13 | 20 | 19 | 0.95  (1.0) | 1.5 | 1.5 |
| 3–4 | 5 | 5 | 14 | 2.80  (1.7) | 1.0 | 2.8 |
| >4 | 7 | 5 | 31 | 6.20 (>2.2) | 0.7 | 4.4 |

The quantitation is of data presented in Fig. 6.
* The numbers in parentheses denote the nucleosome ratio expected if the nucleosome has a 0.75 chance of segregating to the leading strand and a 0.25 chance of segregating to the lagging strand during each pass of a replication fork.

## Nucleosome segregation at the replication fork

To permit a quantitative analysis of nucleosome behaviour in the *in vitro* DNA replication system that we used, we directly photo-crosslinked the replication products containing nucleosomes with psoralen, and examined them by electron microscopy under conditions that normally separate the DNA strands. This method has been used previously to probe chromatin structure[22,40-42]. As expected, when we spread the chromatin templates under these denaturing conditions, they displayed single-stranded bubbles of the size reported for DNA organized with histones in nucleosome structure (Fig. 5b); no such bubbles were observed for the same DNA molecules lacking nucleosomes (data not shown). The chromatin templates used for this experiment were in this way determined to contain an average of three to four nucleosomes.

After replication, the application of this technique revealed rolling-circle DNA molecules with long tails (see Fig. 3a). Both the circle and the tails contained occasional bubbles where a nucleosome had protected the DNA from crosslinking by psoralen (Fig. 5c). These bubbles were of the size expected for nucleosomes (Table 2); as expected, they were only detected when nucleosome-containing DNA molecules were used as the template (data not shown).

We mapped the locations of the bubbles for 46 replicating DNA molecules; the nucleosome distributions inferred are shown in Fig. 6. This figure shows that those molecules that replicated the most extensively tend to have the fewest nucleosomes on their DNA circle and the most on their tail. Because the tail represents the DNA made on the lagging strand of a replication fork, this is the result expected if a nucleosome partitions more or less randomly between the two daughter DNA helices produced at the fork each time that the fork passes.

The 'partition coefficient' for a nucleosome is examined more quantitatively in Table 2 in which the molecules represented in Fig. 6 have been divided into five classes on the basis of the extent of their replication. Because the average number of nucleosomes per DNA molecule does not decrease with an increase in the extent of replication, and is consistent with the number determined for the unreplicated template, we can conclude that most of the histone octamers were inherited intact on the daughter DNA helices. We tabulated the ratio of nucleosomes on the tail to nucleosomes on the circle for each class of replicated DNA molecules (Table 2). Comparison with the theoretical result expected for various partition coefficients indicates that a nucleosome most often segregates to the leading strand, but that it segregates to the lagging strand about one in every four passes of a replication fork (see numbers in parentheses in Table 2). It seems that the choice is made randomly, as it is clear from the presence of nucleosomes all along the DNA tails that the same nucleosome can partition to the leading strand during one pass of the replication fork and then partition to the lagging strand at the next pass of the same replication fork a few minutes later.

## Discussion

### The choice of replication system and DNA template.
We have described here the use of a well-characterized purified prokaryotic DNA replication system to study the behaviour of an important eukaryotic structure, the nucleosome, at a replication fork. The replication proteins used form a multienzyme complex that catalyses concerted leading- and lagging-strand DNA synthesis with *in vivo* rates and fidelities[25]. The choice of a hybrid prokaryotic–eukaryotic system was necessary for our experiments, because no highly purified eukaryotic DNA replication system has so far been developed. But because the system is completely defined, it has the valuable feature of demonstrating that the observed nucleosomal properties are inherent to the nucleosome itself, rather than being due to hypothetical replication proteins that stabilize the histone octamer and tether it to the DNA molecule, thereby preventing its release as the DNA polymerase passes.

By using a specifically nicked circular DNA molecule to produce the artificial chromatin template, we generated replication forks on a high proportion of the template molecules; moreover, once started, these replication forks proceeded around the circle indefinitely, generating the long linear tails diagnostic of the rolling-circle form of DNA replication[39]. A unique advantage of this form of replication is that the extent of replication on each template molecule is readily determined from the tail length measured by electron microscopy. In addition, because the same replication fork passes repeatedly over the daughter DNA helix synthesized on the leading side of the fork, the tail is formed entirely by lagging-strand DNA synthesis. It is this feature of DNA synthesis on a circular template that allowed us to determine how the nucleosome is partitioned between the two daughter DNA helices produced by the replication fork (Fig. 6; Table 2).

### The histone octamer is directly inherited by the daughter DNA helices produced at a replication fork.
The ^{3}H-labelled histones in template-bound nucleosomes co-fractionated with extensively replicated DNA molecules in a manner that is unaffected by the presence of a 10-fold excess of nonreplicating competitor DNA (Fig. 3b and Table 1). Moreover, these histones seem to form normal nucleosomes on the daughter DNA helices: the inherited unit contains all four histones (Fig. 4a), protects ~145 bp of DNA against micrococcal nuclease digestion (Fig. 4b), and masks the same length of DNA against psoralen crosslinking as is masked by native nucleosomes (Table 2). We conclude that nucleosomes can undergo conformational changes that allow replication directly through them. Nucleosomes in solution can adopt different structural states[43,44], and drastic reversible conformational changes of histone–DNA complexes have been observed by electron microscopy[42,45]

Nucleosomes allow *in vitro* transcription to pass them[46,47]. But Lorch *et al.*[46] reported that RNA polymerase displaces the histone octamer from the DNA, whereas Losa and Brown[47] suggest that the histone octamer remains at its initial DNA site

FIG. 4 Characterization of the histones that segregate with replicated DNA in the presence of a large excess of competitor DNA. *a*, Fluorograph of the ³H-labelled histones that elute in peak 1 from a Sepharose 2B gel filtration column. To obtain enough radioactivity for this experiment, the peaks from five separate experiments like that represented in Fig. 3*b* were pooled (lane 2). Because the histones were labelled by the incorporation of ³H-labelled lysine, they had a low specific activity and were relatively difficult to detect. For comparison, lane 1 displays the histones present on the artificial chromatin template before replication. *b*, Analysis of the DNA protected from micrococcal nuclease digestion in the purified replicated chromatin. The material isolated in peak 1 was digested with micrococcal nuclease for the indicated times and its size then analysed by polyacrylamide gel electrophoresis (lanes 3–5). For comparison, the DNA protected from digestion in native core nucleosomes was similarly analysed in lane 1. Lane 2 displays the migration rate of a 125-bp DNA fragment that served as a size marker.
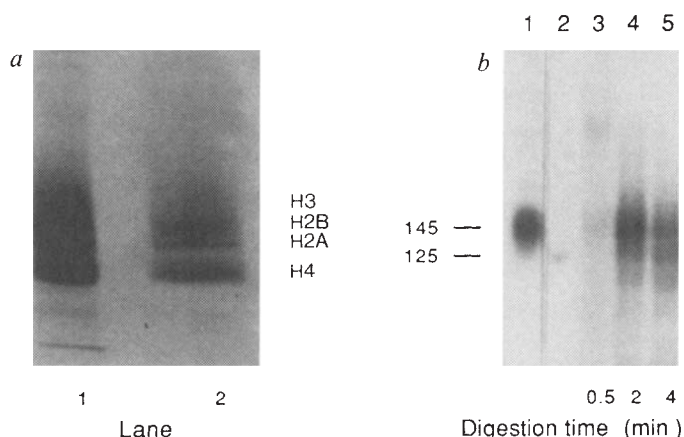


after the RNA polymerase passes. It has previously been suggested that dissociation into half nucleosomes[48] or 'lexosomes'[49] accompanies transcription.

During DNA replication, the entire histone octamer is segregated to only one of the two DNA daughter helices. This implies that the nucleosome core becomes transiently bound to a DNA single-strand, as previously suggested[7,50]. Histone–DNA interactions that are different from those in normal nucleosomes could exist that allow the histones to remain bound transiently to a single DNA strand without covering the nucleotide bases

or interfering with the replication process. Alternatively, the histone octamer could somehow 'rock' back and forth as the DNA polymerase passes, allowing the DNA polymerase to copy a histone-free region of DNA without knocking off the nucleosome completely. In either case, once the replication fork has passed, the histone octamer must re-form a normal nucleosome.

The positions of all of the nucleosomes left on the circular portion of the rolling-circle DNA products in the experiment of Fig. 6 were determined by electron microscopy (data not shown). There was no tendency for these nucleosomes to
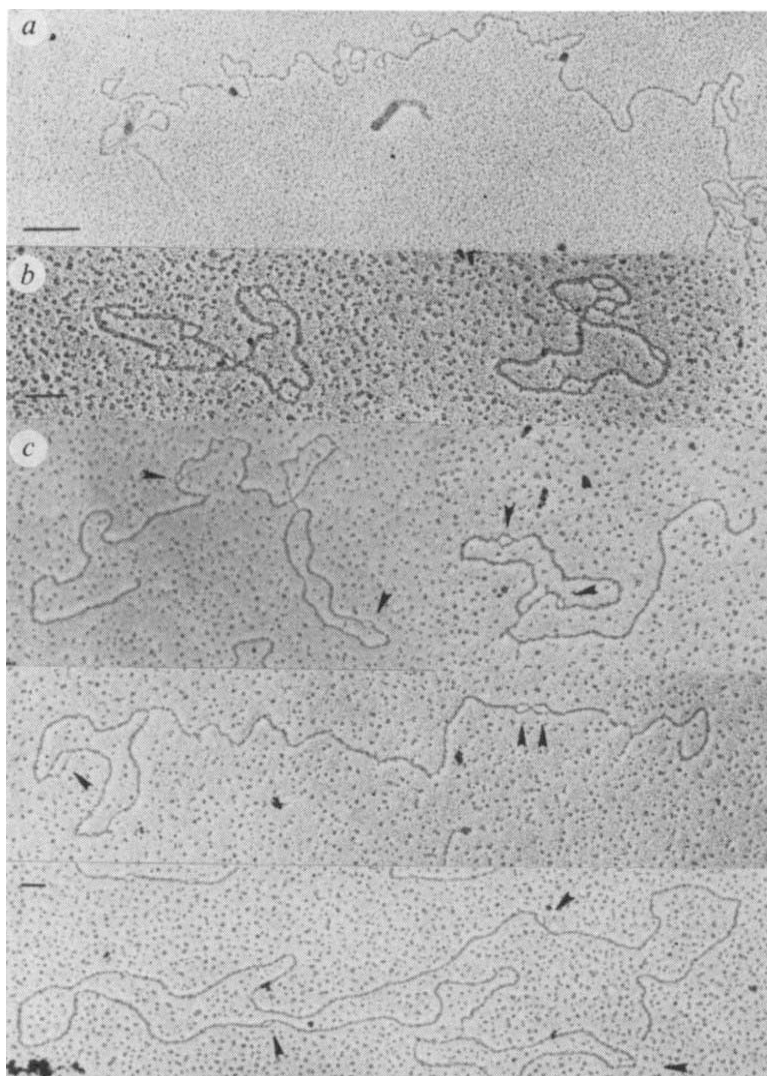


FIG. 5 Electron micrographs of the artificial chromatin template before and after replication. *a*, Replicating molecules spread as chromatin, where nucleosomes appear as small beads (scale bar, 0.1 μm). *b*, Unreplicated chromatin template molecules viewed by the psoralen technique. The positions of nucleosomes appear as single-stranded bubbles in the deproteinized DNA (bar, 0.1 μm). *c*, Four replicating molecules viewed by the psoralen technique. Bubbles representing nucleosomes (arrows) are found on both the DNA circle and its tail (bar, 0.1 μm).

METHODS. The procedure for psoralen cross-linking of H1-depleted chromatin was essentially as described in ref. 63. Samples were mixed with 0.03 μg ml⁻¹ psoralen and then irradiated with ultraviolet light (360 nm) at 4 °C for 65 min (Model UVGL-25 UVP-incorporated lamp) at a distance of ∼2.5 cm. The DNA was then deproteinized by treatment with proteinase K (200 μg ml⁻¹) in the presence of 0.5% SDS for 2 h at 37 °C, followed by two phenol–chloroform extractions and ethanol precipitation. The psoralen-crosslinked DNA was resuspended in 10 mM Tris–HCl buffer, pH 7.4, 1 mM Na₃EDTA, and incubated for 60 min in a denaturing buffer containing 73% (v/v) formamide, 0.5 M glyoxal (Kodak), which separates all noncrosslinked DNA strands. This DNA was then viewed by electron microscopy after spreading, as described in ref. 64.

accumulate near where the tail joins the circle (the fork location). Such an accumulation would be expected if the template-bound nucleosomes tend to be pushed along the DNA template as the replication fork approaches (nucleosome sliding). But whether or not each nucleosome remains on exactly the same DNA sequence after the fork passes cannot be determined from our present results. Further studies using our *in vitro* system should, however, enable us to address this question.

**The parental histone octamer can be transferred to either of the daughter DNA helices when the fork passes.** One side of a replication fork (the lagging strand) contains a single-stranded region of the template not present on the other side (the leading strand). At the T4 replication fork, this single-stranded connection to the parental double helix is often >1,000 nucleotides, and it is normally covered with cooperatively bound gene 32 protein molecules[25]. Intact histone octamers can nevertheless be transferred to the DNA helix made on the lagging strand (the tail in Fig. 6). In one variation of the experiment of Fig. 3*a*, the primase (gene 61 protein) was omitted from the replication reaction so that DNA was synthesized only on the leading side of the fork. In this case, a long length of single-stranded DNA accumulated on the rolling-circle tail because no Okazaki fragments were made. When peak 1 from a gel filtration column like that represented in Fig. 3*b* was analysed, very little ³H-labelled histone was found attached to this replicated DNA (data not shown). This result indicates that the histone octamers that would normally end up on the lagging-strand DNA helix were transferred to the long single-stranded tail instead, where they are relatively unstable[9], and so dissociate during the procedures used to isolate peak 1. Therefore, transfer of a parental histone octamer to the lagging strand does not seem to require the DNA to be double-stranded. We suggest that the normal transfer of the histone octamer to the lagging strand involves a similar unstable intermediate, but because each cycle of Okazaki fragment synthesis requires only a few seconds[25,51], the histone octamer survives to become stabilized as a normal nucleosome on double-stranded DNA.

The presence of an unstable intermediate during nucleosome transfer from parental to daughter DNA can explain why the regions of newly synthesized DNA *in vivo* (as measured by pulse-label incorporation of ³H-labelled thymidine) have sometimes been found to be free of nucleosomes after chromatin extraction[15,52,53]. In most of these experiments, the chromatin was washed in 200 mM NaCl before analysis, and there is evidence that the histones on newly replicated DNA are unusually sensitive to salt-induced dissociation[54]. In addition, Sogo *et al.*[22] failed to detect nucleosomes both just in front of the replication fork and just behind it when the psoralen crosslinking technique was applied to SV40 chromatin replication *in vivo*. Although their results were interpreted as indicating that the histone octamer is released from the DNA as the replication fork approaches, our results support the alternative view that the histones remain on the DNA, but are present in an altered more-open complex that leaves the DNA accessible to psoralen crosslinking[42]. The nature of this altered form of the nucleosome could be susceptible to analysis in our *in vitro* system.

**The partition coefficient for histone octamers between leading- and lagging-strands.** Our results indicate that although the histone octamers ahead of the fork can remain associated with either of the strands at the replication fork, each shows a bias of about 3 to 1 towards remaining on the leading strand (Fig. 6 and Table 2). Given the inherent difficulty of analysing the process *in vivo*, it is perhaps not surprising that the results of direct nucleosome segregation studies on cells have been the subject of intense controversy. Most recent studies tend to support the claim that the parental histones are distributed to both sides of the replication fork *in vivo*[2]. But our *in vitro* results are in agreement with those of a recent study[14] indicating that the segregation of nucleosomes is biased in favour of the leading strand in cycloheximide-treated mammalian cells (in these studies, as in ours, no new nucleosomes were deposited behind the fork).

Our *in vitro* system is not a perfect mimic of the eukaryotic replication fork moving through native chromatin. First, the artificial chromatin template that we used contains only about one nucleosome per kb, a five-fold lower density of nucleosomes than is present *in vivo*. Second, the nucleosomes that we used were randomly distributed along the DNA template, rather than organized into regular repeated arrays with the aid of histone H1. Third, it is possible that special eukaryotic proteins—either replication proteins or non-histone proteins with a structural role—influence the behaviour of the histone octamer at the fork, perhaps even causing different patterns of nucleosome segregation in different regions of the genome. Finally, in eukaryotic cells the Okazaki fragments (and therefore the single-stranded regions of template on the lagging strand) are much shorter than they are in our prokaryotic replication system (averaging 200 nucleotides rather than ~1,500 nucleotides), and the T4 gene 32 protein is, of course, not present on the single-stranded regions of the lagging-strand. In our view, these differences could affect the partition coefficient for a nucleosome between leading and lagging strands, but they are unlikely to change the basic conclusions derived from our *in vitro* study.

## Conclusions

Our main conclusions are that the histone octamers remain bound on the DNA template when a replication fork passes and that they are designed in a way that allows them to transfer
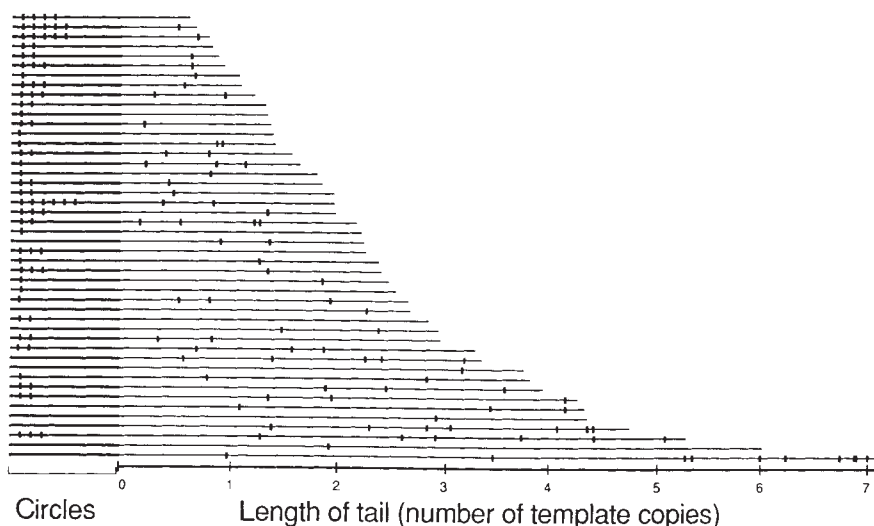
FIG. 6 Histogram showing the positions of nucleosomes on 46 replicating DNA molecules, measured from micrographs like those shown in Fig. 5c. The artificial chromatin template circles used for this experiment contained an average of three to four nucleosomes. Replication was for 10 min at 37 °C. The short vertical bars show where the centres of nucleosomes were located on the tails of each rolling-circle molecule; by contrast, the bars on the circular portions of each molecule denote the number, but not the position, of nucleosomes. For a schematic diagram of these replicating molecules, see Fig. 3a.

Circles    Length of tail (number of template copies)

efficiently to either one of the two daughter DNA helices produced by the fork. Because the basic mechanisms of DNA replication and the geometry of the replication fork seem to be identical for prokaryotes and eukaryotes[55], the results that we obtained with the T4 bacteriophage model system have important implications for eukaryotic replication. Even though replication over nucleosomes would seem to demand significant conformational changes in the histone octamer, the observation that it can be achieved without eukaryotic proteins demonstrates that histones are especially designed to facilitate this important biological process. We suggest that the striking evolutionary conservation of the histone amino-acid sequences, as well as the construction of the nucleosome core from eight small histone molecules, reflects the need for transient nucleosome structures that allow the DNA in eukaryotic chromosomes to be condensed and packaged without interfering with either DNA replication or transcription. □

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# Large-scale distribution of galaxies at the Galactic poles

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GALAXIES, mapped in two or three dimensions, are not distributed randomly but are clustered on small scales ($<5\ h^{-1}$ Mpc, where $h \approx 0.5{-}1$ is Hubble's constant in units of $100\ \mathrm{km\ s^{-1}\ Mpc^{-1}}$), for reasons conventionally ascribed to the effects of gravity. Whether galaxies remain correlated on very large scales ($\sim 50{-}100\ h^{-1}$ Mpc) is of particular interest, because such structures are unexpected in most cosmological theories. We have combined data from four distinct surveys at the north and south Galactic poles to produce a well sampled distribution of galaxies by redshift on a linear scale extending to 2,000 $h^{-1}$ Mpc. Here we report our finding of an excess correlation and an apparent regularity in the galaxy distribution with a characteristic scale of 128 $h^{-1}$ Mpc. This structure is revealed only after the completion of recent surveys extending to redshift $z > 0.2$. Similarly deep surveys with greater angular spread are needed to verify our results and to determine the implications for cosmology.

Recently there has been a considerable increase in information on the three-dimensional distribution of galaxies from large systematic redshift surveys. These surveys have generally been of two kinds. First, there are wide-angle shallow surveys[1] in which information on the clustering is affected by problems of non-uniform detection and questions of whether the local volumes probed are representative. Second, there are deeper narrow-cone surveys in a few directions[2-4]; these overcome such problems to some extent but require more telescope time. Recently, through the advent of multiple-object spectroscopy, some very deep pencil-beam surveys have been attempted[5,6], the aim of which is to study collectively galaxy evolution and clustering on substantially larger scales than hitherto possible.

Much of the pencil-beam work has concentrated at the two Galactic poles, where effects of obscuration should be minimal. A deep-redshift survey recently completed at the Anglo-Australian telescope (AAT) sampled five southern high-latitude fields of 20-arcmin diameter to a limiting photographic magnitude $b_J = 21.5$ (ref. 5) ($b_J =$ Kodak IIIa − J + Schott GG385). Independently, at Kitt Peak, a second long-term redshift survey